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Optimization of Adsorptive Immobilization of Alcohol Dehydrogenases

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In this work, a systematic examination of various parameters of adsorptive immobilization of alcohol dehydrogenases (ADHs) on solid support is performed and the impact of these parameters on immobilization efficiency is studied. Depending on the source of the enzymes, these parameters differently influence the immobilization efficiency, expressed in terms of residual activity and protein loading. Residual activity of 79% was achieved with ADH from bakers' yeast (YADH) after optimizing the immobilization parameters. A step-wise drying process has been found to be more effective than one-step drying. A hypothesis of deactivation through bubble nucleation during drying of the enzyme/glass bead suspension at low drying pressure (<45 kPa) is experimentally verified. In the case of ADH from *Lactobacillus brevis* (LBADH), >300% residual activity was found after drying. Hyperactivation of the enzyme is probably caused by structural changes in the enzyme molecule during the drying process. ADH from *Thermoanaerobacter* species (ADH T) is found to be stable under drying conditions (>15 kPa) in contrast to LBADH and YADH.

[**Key words:** yeast alcohol dehydrogenase, *Lactobacillus brevis* alcohol dehydrogenase, *Thermoanaerobacter* species alcohol dehydrogenase, immobilization, physical adsorption, protein loading]

The production of ketones and optically active alcohols using alcohol dehydrogenases (ADHs) in aqueous solutions has been carried out by a number of investigators (1, 2). Unfortunately the operational instability of ADHs in aqueous solution limits their widespread industrial application (3, 4). Application of ADHs in non-conventional media such as organic solvents and in a gas/solid system (enzymatic gas phase system) could overcome some of the problems associated with the ADH instability in aqueous media (5, 6). However, the use of enzymes in powder form in non-conventional media may lead to mass transfer limitations. The multiple use of such enzymes is almost impossible. This problem can be minimized by immobilization of the enzyme on support particles.

The immobilization of alcohol dehydrogenase on various supports has been investigated by several researchers (4, 5, 7–9). Commonly used procedures are adsorption or covalent linkage to a carrier. Covalent coupling is not necessary in non-conventional media, since the proteins are generally insoluble in this media. Moreover, immobilization of enzyme by physical adsorption (deposition) provides a simple means for retention in continuous reactors (10, 11). Often, an increased thermostability of the catalyst after adsorption is observed (12–14). However, the reported, still quite significant losses in enzyme activity during the immobilization by physical adsorption (15) leave room for optimization, which is especially required for expensive enzymes. Various studies have investigated several immobilization parameters such as pH, ionic strength, concentration of enzyme or application of salts (7, 14–17), resulting in enhanced residual activities.

In contrast, the effect of other parameters of the immobilization procedure, *i.e.*, the stirring temperature, time, speed, addition of sucrose, and drying temperature and pressure are seldomly analyzed. Therefore, in this work a careful investigation of these parameters and their impact on the residual activity and protein loading is presented. Based on the results of this study, a procedure with optimized parameters was obtained resulting in markedly enhanced residual activities.

Yeast alcohol dehydrogenase (YADH) was chosen as model enzyme, because of its known high sensitivity and poor stability (3, 4), which make it a suitable candidate for the present study. In the present work, non-porous solid glass beads (0.25–0.30 mm) were selected as a support because of their mechanical, chemical and thermal stability (18). Previous investigation showed that the small size and non-porosity reduce the diffusional constraints with respect to substrate and product in immobilized systems (19). Due to their defined geometry, this support material served as a reference to understand the general mechanism during the preparation of the immobilized enzyme. At this step, it was not the aim to maximize the surface area. In addition to this support, other non-porous supports such as Celite 545, Celite 503 (both are silicic acid-based supports) and Poly-

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amide at optimal immobilization conditions were studied. The key results have been applied to two industrially relevant stereoselective ADH enzymes, namely *R*-specific ADH from *Lactobacillus brevis* (LBADH) and *S*-specific ADH from *Thermoanaerobacter* species (ADH T).

These ADH enzymes can be used as biocatalysts in non-conventional media by co-immobilizing the ADH with its cofactor on the support. Therefore, the regeneration of the cofactor (NADH or NADPH) has to be carried out using a co-substrate. For alcohol dehydrogenases, short chain aliphatic alcohols are typically applied (5, 6). Initial results on the application of immobilized LBADH in gas-phase catalysis using substrate-coupled cofactor regeneration are reported in the paper of Ferloni *et al*. (6).

MATERIALS AND METHODS

Enzyme, support, and other chemicals Lyophilized alcohol dehydrogenase (EC 1.1.1.1) from bakers' yeast (YADH) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). It has a specific activity of 330 units/mg protein measured at 22°C. The protein purity was 0.55 mg/mg of lyophilized powder. Lyophilized LBADH (EC 1.1.1.2) (*Lactobacillus brevis* alcohol dehydrogenase containing sucrose at 5 times the amount of protein [w/w]) was obtained from Juelich Fine Chemicals (Juelich, Germany). The protein purity was 0.17 mg/mg of lyophilized powder with a specific activity of 89 units/mg protein. Lyophilized ADH T (*Thermoanaerobacter* species alcohol dehydrogenase containing sucrose at 5 times the amount of protein [w/w]) was obtained from Juelich Fine Chemicals. The protein purity was 0.13 mg/mg of lyophilized powder with a specific activity of 86 units/mg protein measured at 30°C. β-Nicotinamide adenine dinucleotide, oxidized form (β-NAD^+) and β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH) were purchased from Biomol (Hamburg, Germany).

Non-porous glass beads (0.25–0.30 mm) were supplied by B. Braun Biotech International (Melsungen, Germany). Non-porous Celite 545 (0.01–0.02 mm) and non-porous Celite 503 (<0.1 mm) were obtained from Merck (Darmstadt, Germany), non-porous Polyamide particles (0.05–0.16 mm) from Carl Roth (Karlsruhe, Germany). Celites and Polyamide were pre-wetted with 50 ml of ethanol for 30 min and then pre-washed with a 50% (v/v) ethanol–water solution. Then, the mixture was washed several times with distilled water and the wet beads were used for immobilization.

Ethanol, acetophenone, magnesium chloride, and tris(hydroxylmethyl)-aminomethane hydrochloride (Tris) were bought from Merck. Triethanolamine-hydrochloride (Tea) and potassium dihydrogen phosphate (PO_4) were from Fluka Biochemika (Steinheim, Germany). Bradford reagent and lyophilized bovine serum albumin (BSA) powder were supplied by Bio-Rad Laboratories (Munich, Germany), while silica gel orange and acetone were from Carl Roth. All other reagents and salts were of analytical grade.

Immobilization method Unless otherwise stated, 1 ml of 100 mM PO_4-buffer (pH 7) containing 4 mg of dissolved YADH was added to a 25-ml beaker containing 500 mg of carrier at 4°C. The resulting mixture was stirred for 2 h with a magnetic stirrer (20 mm length and 6 mm diameter) at 125 ± 5 rpm. Then, the beaker was placed in a desiccator containing silica gel orange and the whole enzyme/support suspension was dried at 4°C under an absolute pressure of 15 kPa (unless otherwise specified) until all the water was removed from the preparation (see setup in Fig. 1). In this manner, free flowing support was obtained. The time required for drying was recorded. The dried immobilized enzyme preparation was removed from the beaker and finally stored at 4°C. It should be noted that some of the enzyme remained in the beaker depending on the adsorptive affinity of the enzyme to the support material. Similarly, the immobilization of LBADH was performed using 50 mM Tea-buffer (pH 7) containing 1 mM $MgCl_2$ and 2 mg of protein. For immobilization of ADH T, 50 mM Tris-buffer (pH 7) and 2 mg of protein was used. Immobilization of ADHs from different sources was performed in the buffers at the concentrations supplied by the manufacturers for the enzyme preparation as standard.

With the view of future application of the ADH enzyme in non-conventional media, immobilization of ADH with and without addition of cofactor was performed. A cofactor to protein ratio of 0.01 (w/w) was used for immobilization. Preliminary investigation showed almost no impact of cofactor addition on the residual activity analyzed in aqueous solution (data not shown). Hence, only the studies on immobilization parameters without addition of cofactor are presented.

Protein determination The Bradford method (20) was used to measure the protein concentration of solutions with dissolved protein at 595 nm with a UVIKON 922 spectrophotometer (Kontron Instrument, Milano, Italy), using bovine serum albumin (BSA) as a standard. The amount of protein loaded onto the dried support was determined according to the method described by Bonde *et al*. (21). The assay measures the decrease in the absorbance of the solution at 465 nm due to adsorption of dye by the bound protein. In contrast with the usual Bradford method, this assay does not measure the absorbance of the soluble protein-dye complex at 595 nm and thus is referred to as the 465 Bradford assay (21). For this assay, which is basically a modified Bradford method, 200 µl of distilled water were added to 4.5 mg of glass beads loaded with enzyme as well as to 4.5 mg of blank glass beads, respectively. Bradford reagent (1800 µl), diluted (1:5) with distilled water, was subsequently added to each of these mixtures. After brief agitation of the mixture (3 min) to allow binding of the dye to the protein, the mixtures were centrifuged at 18,000 × *g*. The absorbance of the two supernatants was measured at 465 nm and the reading obtained with the blank support was subtracted from the reading obtained from the loaded support. The calibration curve for quantification of the protein was obtained with BSA as a reference protein. The protein loading on the dried support was calculated as the ratio of mg of protein loaded on the support to the total mg of support used.

Assay of enzyme activity The activity of lyophilized YADH enzyme (before immobilization) was determined at 22°C by adding 20 µl of a 12.5 mM β-NAD^+ cofactor solution to 970 µl of 100 mM PO_4-buffer (pH 7.84) containing 100 mM ethanol as a substrate. To initiate the reaction, 10 µl of the enzyme solution were added to the mixture. The increase in absorption at 340 nm due to the formation of NADH was measured with a spectrophotometer. A molar extinction coefficient for reduced cofactor of $6.22 \cdot 10^{-3}$ $l \cdot \mu mol^{-1} \cdot cm^{-1}$ was used for calculation of the enzyme activity. One unit of ADH enzyme activity was defined as the amount of enzyme required to convert (reduce or oxidize) 1 µmol of cofactor per min under the experimental conditions. By using this method, the activity of YADH before immobilization was found to be 330 units/mg protein. The activities of the lyophilized LBADH and ADH T were measured in a similar manner. For the assay of LBADH activity, 9.5 mM β-NADPH in 50 mM Tea-buffer (pH 7) containing 1 mM $MgCl_2$ and 11 mM acetophenone at 30°C was used. For ADH T, 10 mM β-NADPH containing 50 mM Tris-buffer (pH 7) with 10 mM acetone at 30°C was used. The activities of LBADH and ADH T were found to be 89 units/mg protein and 86 units/mg protein, respectively. In a similar manner, the residual activity of the immobilized ADHs was measured in aqueous solution by redissolving the immobilized enzyme in 100 ml of buffer with their respective substrates and cofactor, as described for the activity de-

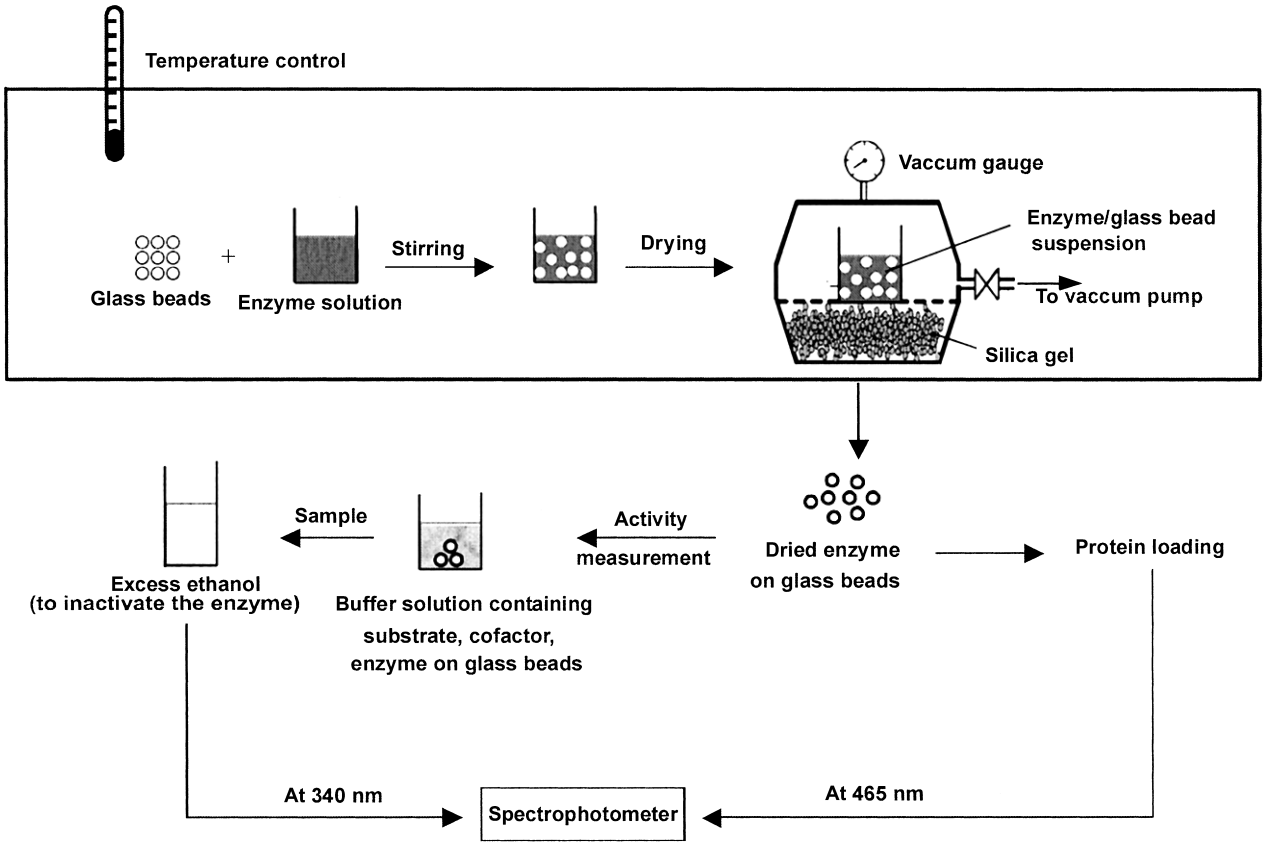

FIG. 1. Schematic drawing of experimental setup for immobilization of enzyme.

termination of the lyophilized enzymes. The reaction mixture was stirred at 350 rpm for 10 min. Samples of 500 µl were periodically withdrawn and added into 500 µl of ethanol in order to inactivate the enzyme. In a preliminary experiment, it was verified that all three enzymes were inactivated by the resulting concentration of ethanol. The samples were then analyzed by measuring the change in absorption of the reduced nicotinamide cofactor at 340 nm. The initial linear portion of the curve was used to determine the activity of the immobilized enzymes. Finally, the residual activity of ADHs was calculated as a ratio of the activity measured after immobilization in units/mg protein to the activity before the immobilization in units/mg protein and expressed as a percentage.

RESULTS AND DISCUSSION

Effect of temperature on immobilization efficiency of YADH Three combinations of stirring and drying temperature for immobilization of YADH were chosen. Figure 2 shows the results. A low residual activity (30%) was obtained when both stirring and drying were done at high temperature (22°C), while stirring at low temperature (4°C) and drying at high temperature (22°C) resulted in a high residual activity (48%). This activity was further increased to 60%, when the stirring and drying were carried out at a low temperature (4°C). Thus, low temperature was found to be less detrimental to the enzyme during stirring and drying. This observation is in line with that of Liao and Chen (8), who explained that this behavior was due to reduced thermoinactivation of the enzyme at lower temperature. However,

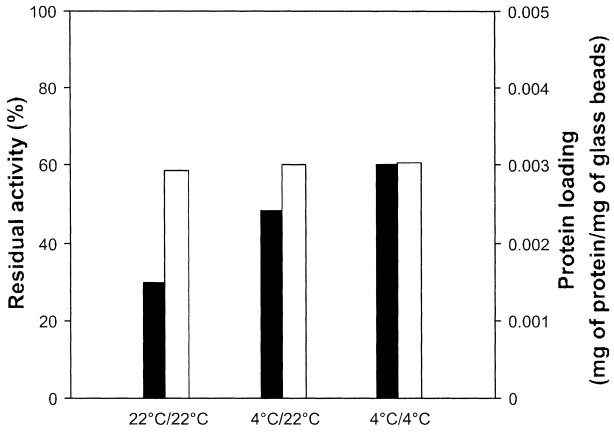

FIG. 2. Effect of temperature on immobilization efficiency of YADH. Black bars represent the residual activity and white bars denote the protein loading. Conditions: 4 mg of lyophilized YADH, 500 mg of glass beads, 1 ml of 100 mM PO_4-buffer (pH 7.84), 2-h stirring time, 125±5-rpm stirring speed, 15 kPa absolute drying pressure.

Fig. 2 does not reveal a notable change in the protein loading on the glass beads with different stirring and drying temperatures. The protein loading was found to be 0.03 mg per mg of non-porous glass beads.

Effect of stirring speed and time on immobilization efficiency of YADH In a preliminary experiment, it was found that the unstirred sample had non-uniform protein

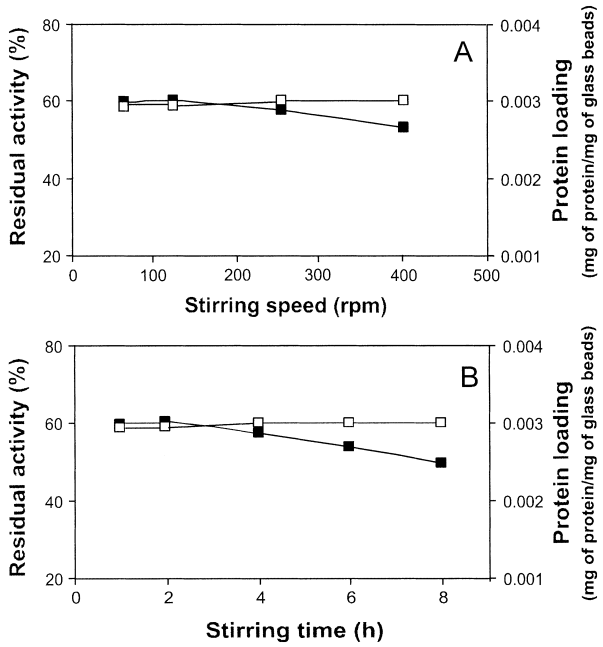

FIG. 3. Effect of stirring speed (A) and stirring time (B) on immobilization efficiency of YADH. Closed squares represent the residual activity and open squares denote the protein loading. Conditions: 4 mg of lyophilized YADH, 500 mg of glass beads, 1 ml of 100 mM PO_4-buffer (pH 7.84), 4°C temperature, 15 kPa absolute drying pressure, (A) 2-h stirring time, (B) 125±5-rpm stirring speed.

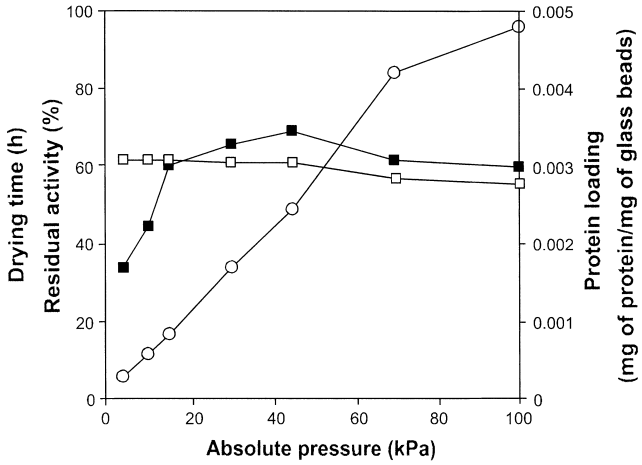

FIG. 4. Effect of pressure during drying on immobilization efficiency of YADH. Closed squares represent the residual activity, open squares denote the protein loading, and open circles represent the drying time. Conditions: 4 mg of lyophilized YADH, 500 mg of glass beads, 1 ml of 100 mM PO_4-buffer (pH 7.84), 4°C temperature, 2-h stirring time, 125±5-rpm stirring speed. (The standard deviation of the residual activity was approximately 1.5%.)

loading on the glass beads. Therefore, immobilization of YADH on non-porous solid glass beads was performed at four different stirring speeds (50, 150, 250, 400 rpm) keeping the adsorption time (2 h) constant. The results are shown in Fig. 3A. It was observed that the residual activity decreased with increasing stirring speed. This may be attributed to detrimental shearing effects on the enzyme caused by the magnetic stirrer as previously reported by several researchers (22, 23). Preliminary investigations showed that a stirring time shorter than 1 h resulted in non-uniform protein loading on the glass beads. Exposure of the enzyme to prolonged stirring time reduced its activity, as shown in Fig. 3B. This may be also due to the shear forces induced by the stirrer, as reported previously (22, 23). In Fig. 3, the protein loading of the glass beads was very similar to that shown in Fig. 2 and remained almost constant with different stirring speeds and times.

Effect of drying conditions on immobilization efficiency of YADH Dehydration is a known stress to proteins potentially causing protein unfolding (24). Thus, a detailed investigation of the drying process and its effects on residual activity as well as on protein loading was conducted. Drying of the enzyme/glass bead suspension was performed at seven different absolute pressures starting from atmospheric pressure (100 kPa) down to 4 kPa. The results are shown in Fig. 4. The time required for drying of the preparation was significantly reduced with a decrease in absolute pressure. This can be explained by the inverse relationship between the diffusion coefficient and pressure (25), which resulted in enhanced mass transfer from the aqueous solution to the silica gel at low pressure (cf. Fig. 1). A slight decrease in protein loading with increasing absolute pressure was observed. A very interesting relationship between the drying pressure and the residual activity was found. With decreasing absolute pressure from atmospheric pressure, the residual activity increased until a pressure of 45 kPa was reached. At lower pressures, the residual activity decreased. At the optimum pressure (45 kPa), the residual activity was found to be 69%. Interestingly, this value is almost 7% higher than the highest residual activity (62%) reported to date in the literature for immobilized YADH (8). A tentative explanation behind the low residual activity obtained at higher absolute pressure resulting in extended drying time can be obtained from Lui *et al.* (26). According to those authors, prolonged drying of some enzymes at high absolute pressure causes inter-molecular S-S bond formation via thiol-disulfide interchange reactions of cysteine residues, which results in loss of enzyme activity. However, low residual activity was also obtained at a lower pressure and this effect was critically studied. By visual inspection of the enzyme/glass bead suspension during the drying process, it was found that air dissolved in the solution formed bubbles. Bubble nucleation could have a detrimental effect on enzyme activity. In order to investigate the effect of this bubble nucleation on the immobilization efficiency, another experiment was conducted, whereby the drying process of the enzyme preparation was divided into two steps. In the first step, drying was performed at 45 kPa for 3 h in order to gently remove the dissolved air from the solution. In the second step, drying was carried out at 4 kPa for 9 h. As a result of this, 71% residual activity was achieved, as shown in Fig. 5. Compared to drying at a constant pressure of 45 kPa, drying time was significantly reduced. To achieve this, one more experiment was conducted, where after the typical 2-h stirring of the enzyme/glass bead suspension, helium was pumped through the gaseous headspace of the closed bottle for 30 min in order to strip off the air from the solution. This stripping was achieved by the equilibration of the helium

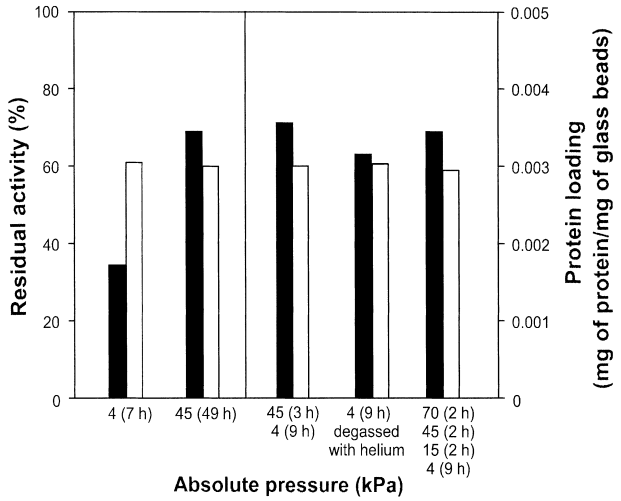

FIG. 5. Effect of drying conditions on immobilization efficiency of YADH. Black bars represent the residual activity and white bars denote the protein loading. The conditions were equivalent to those described in Fig. 4. The values in parentheses indicate the hours employed for drying at the specific pressure.

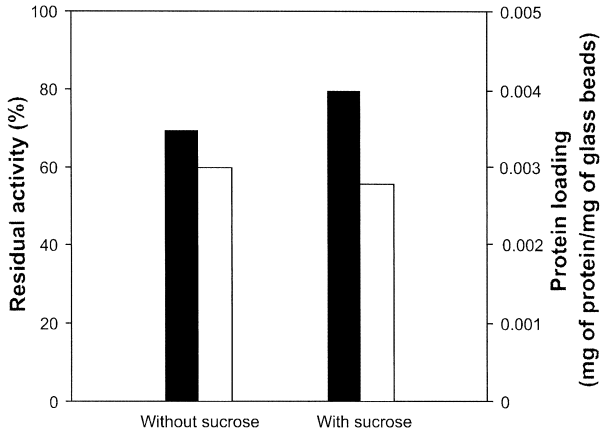

FIG. 6. Effect of sucrose addition on immobilization efficiency of YADH. Black bars represent the residual activity and white bars denote the protein loading. Conditions: 4 mg of lyophilized YADH, 500 mg of glass beads, 1 ml of 100 mM PO_4-buffer (pH 7.84), 4°C temperature, 2-h stirring time, 125±5-rpm stirring speed, 45 kPa absolute drying pressure, with sucrose: sucrose 5 times the protein amount (w/w) was added.

gas phase and the water phase during the employed period of time. Since helium has a low solubility in water compared to oxygen and nitrogen (27), only a significantly reduced amount of dissolved gas (helium) was finally present in the aqueous solution. After this degassing step, the solution was subsequently dried at 4 kPa absolute pressure. As shown in Fig. 5, a residual activity of 63% was found compared to the residual activity of 34% obtained with the same pressure without preliminary degassing of the enzyme/glass bead suspension. This gain in residual activity confirms that the bubble nucleation was indeed responsible for the reduced residual activities at low pressure.

In order to test whether more steps in the drying process could further minimize the bubble nucleation, the drying of the enzyme preparation was performed in several steps: the first step was at 70 kPa for 2 h, followed by 45 kPa for 2 h, then 15 kPa for 2 h, while the remainder of the drying was continued at 4 kPa. This experiment resulted residual activity of 69%, as shown in Fig. 5. Almost the same residual activity was obtained in a one-step drying process at 45 kPa and in the two-step drying as shown in Fig. 5, respectively. Thus, the two-step drying process is not only effective in retaining high residual activity but also significantly decreases the drying time as compared to the one-step and multiple-step drying processes.

Effect of sucrose addition on the immobilization efficiency of YADH With a view to the stabilizing effect of sucrose, which forms an amorphous phase with the protein and hydrogen bonds in place of water (28), an experiment was conducted whereby an amount of sucrose equal to 5 times the amount of protein (w/w) was added to the protein/glass bead suspension before the drying process was performed at 45 kPa absolute pressure (Fig. 6). This experiment gave a residual activity of 79%, which is almost 17% higher than the highest residual activity (62%) reported in the literature to date for immobilized YADH (8). Thus, the significant role of sucrose in the protection of protein during drying and storage is again proved (28, 29). Protein loading was slightly reduced when sucrose was added. Obviously, sucrose occupies the adsorption sites on the support material and consequently less sites on the support are available for protein binding. Ferloni *et al.* (6) have shown that enzyme preparations prepared with sucrose have markedly improved stability under reactive gas phase conditions.

Effect of various supports on immobilization efficiency of YADH Apart from the non-porous solid glass beads, immobilization of YADH was also conducted on other non-porous supports, such as Celite 545 (0.01–0.02 mm), Celite 503 (<0.1 mm) and Polyamide (0.05–0.16 mm) at the optimal immobilization conditions with and without addition of sucrose. It was the aim of this experiment to investigate the influence of different surface properties of the support material on the immobilization efficiency. The results are shown in Table 1. As observed, sucrose addition generally enhanced the residual activity and decreased the protein loading of YADH enzyme on all supports. This effect was most pronounced in the case of Celite 545. The reason for the decrease in protein loading due to the addition of sucrose is given in the previous paragraph. Assuming all support particles to be spherical, the ratio of the surface area of the support to the total available surface area for adsorption (support and beaker used during the drying process) was calculated. For example, in the case of non-porous glass beads, this ratio was found to be 0.7. If the affinity of the enzyme towards the support particles and the inner wall of the beaker is equivalent, this implies that about 70% of the protein in solution (which amounts to 0.0056 mg of protein/mg of support) would be adsorbed on the support and the remainder of about 30% would be adsorbed on the inner wall of the beaker. The calculated amount of protein loading in mg per mg of support is termed the theoretical protein loading. For all of the supports, the experimental protein loading was lower than the theoretical value. In all cases, after drying, dry enzyme powder together with salt crystals was found that did not adsorb either onto the support or the inner wall

TABLE 1. Effect of various supports on immobilization efficiency of YADH

Support	Treatment	Particle size (mm)	Residual activity (%)	Theoretical protein loading[a] (mg protein/mg support)	Experimental protein loading (mg protein/mg support)
Glass beads	Without sucrose	0.25–0.30	69	0.0056	0.0030
	With sucrose		80		0.0027
Celite 545	Without sucrose	0.01–0.02	59	0.008	0.0055
	With sucrose		83		0.0043
Celite 503	Without sucrose	<0.1	53	0.0079	0.0047
	With sucrose		68		0.0042
Polyamide	Without sucrose	0.05–0.16	58	0.0078	0.0025
	With sucrose		72		0.0022

[a] Theoretical protein loading is calculated using the average size of the non-porous support material.

of the beaker. Scanning electron microscopic pictures revealed that the support particles were uniformly covered by a homogeneous layer of protein (enzyme) and sucrose. The ratio between the experimental to the theoretical protein loading was significantly lower in the case of polyamide compared to the other supports. This clearly indicates that the surface property of the support material also plays an important role. Accordingly, among these various supports, Celite 545 retained the highest protein loading of 68.8% without sucrose addition. For the two Celite preparations with the same surface characteristics, the protein loading increased with decreasing particle size as previously stated by Bailey and Cho (30).

Effect of pressure during drying on immobilization efficiency of LBADH As with YADH, the immobilization and drying of LBADH was also carried out under various pressures. The results are shown in Fig. 7A. With LBADH, the maximum residual activity, reaching 316%, was also achieved at a drying pressure of 45 kPa. Interestingly, the residual activity of the LBADH at all pressures was markedly higher than that of the native enzyme at all pressures. In regard to the hyperactivation of lipases by selective interfacial adsorption, as mentioned by Bastida *et al.* (31), we hypothesize that the adsorptive immobilization of LBADH might lead to some structural changes in the enzyme molecule, which were retained even after redissolving the immobilized enzyme in aqueous solution during activity determination. In the case of LBADH, there was a slight decrease in protein loading with increasing drying pressure. At low pressures, about 40% of the protein initially added during the immobilization procedure was found adsorbed on the glass beads in contrast to 35% for YADH. It should be mentioned that these percentages were constant for different protein concentrations (up to 0.015 mg per mg/glass beads) in the initial enzyme solution before drying (data not shown).

Effect of pressure during drying on immobilization efficiency of ADH T Figure 7B shows the residual activity behavior of ADH T at different drying pressures. From atmospheric pressure down to 15 kPa almost a 100% recovery of enzyme activity was observed. Only at the lowest pressure (4 kPa), was a significant decrease in residual activity found. The reason most probably is again due to bubble nucleation under this condition. This indicates that ADH T is more stable at drying pressures of 15–45 kPa as compared to LBADH and YADH enzymes. The protein loading behaviors of ADH T and LBADH were very similar. The theoretical protein loading of these two ADHs was 0.0028 mg per mg of glass beads. About 70% of the theoretical protein loading was loaded on the support for each of these proteins.

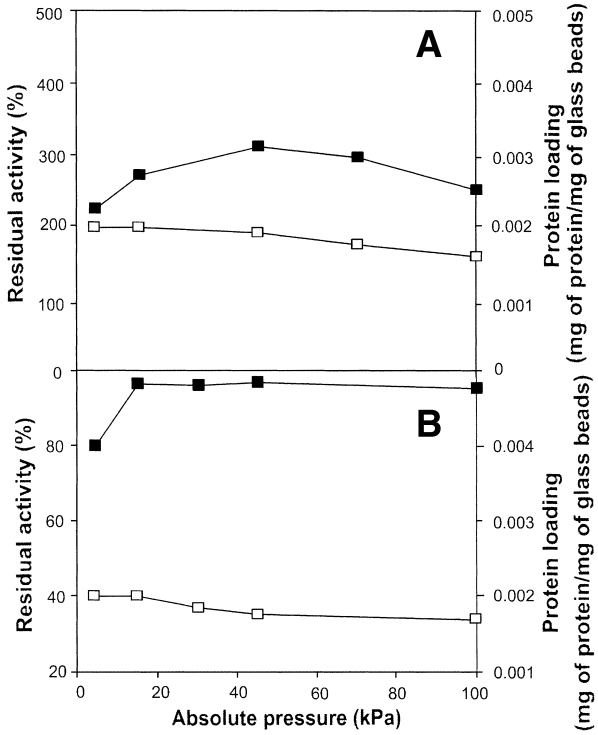

FIG. 7. Effect of pressure during drying on immobilization efficiency of LBADH (A) and ADH T (B). Closed squares represent the residual activity and open squares denote the protein loading. Conditions for immobilization of ADH: 2 mg of lyophilized ADH, 500 mg of glass beads, in the case of LBADH, 1 ml of 50 mM Tea-buffer (pH 7) containing $MgCl_2$, in the case of ADH T, 1 ml of 50 mM Tris-buffer (pH 7), 4°C temperature, 2-h stirring time, 125±5-rpm stirring speed.

Conclusion The following results are evident from this research work: Low drying temperature and mild stirring conditions resulted in increased residual activities, while the protein loading of glass beads remained unchanged. Bubble nucleation had a detrimental effect on the residual activity of ADHs at pressures lower than 45 kPa. A residual

activity of 79% was achieved following optimization of the immobilization parameters in the case of YADH, which is superior to residual activities reported in the literature (8). There was a slight decrease in protein loading with increasing drying pressure. The addition of sucrose increased the residual activity of YADH on all of the supports, while the protein loading on the supports was decreased due to the decrease in the number of free adsorption sites on the support for protein loading. The percentage of YADH protein loaded on the support was in the range of about 35% to 68% and that of LBADH and ADH T was about 40% to 50% relative to the initially added protein depending on the immobilization conditions. The residual activity and the protein loading behaviors of the investigated ADHs at different drying pressures were found to be different depending on the adsorptive affinity of the protein to the support material.

The hyperactivation of the immobilized LBADH might be due to structural modification during immobilization, which was retained even after redissolving the immobilized enzyme in aqueous solution during its activity determination. Among the three ADHs, the thermostable ADH T was found to be more stable at low drying pressures of 15 to 100 kPa, compared to other studied ADHs. The methods reported in this work may be helpful for drying other enzymes on solid supports. In particular, the described optimization of drying pressure is believed to be valuable and generally applicable for different biocatalysts. Details on the performance of the immobilized ADH enzymes in non-conventional media, especially in gas-phase reactions, will be presented in a future work.

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